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## Tissue-specific alterations of methyl group metabolism with DNA hypermethylation in the Zucker (type 2) diabetic fatty rat.


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## Abstract

**Background** Altered methyl group and homocysteine metabolism were tissue-specific, persistent, and preceded hepatic DNA hypomethylation in type 1 diabetic rats. Similar metabolic perturbations have been shown in the Zucker (type 2) diabetic fatty (ZDF) rat in the pre-diabetic and early diabetic stages, but tissue specificity and potential impact on epigenetic marks are unknown, particularly during pathogenesis.

**Methods** ZDF (*fa/fa*) and lean (+/?) control rats were killed at 12 and 21 weeks of age, representing early and advanced diabetic conditions. Blood and tissues were analysed with respect to methyl group and homocysteine metabolism, including DNA methylation.

**Results** At 12 weeks, hepatic glycine *N*-methyltransferase (GNMT), methionine synthase, and cystathionine  $\beta$ -synthase (CBS) activity and/or abundance were increased in ZDF rats. At 21 weeks, GNMT activity was increased in liver and kidney; however, only hepatic CBS protein abundance (12 weeks) and betaine-homocysteine *S*-methyltransferase mRNA expression (21 weeks) were significantly elevated (78 and 100%, respectively). Hepatic phosphatidylethanolamine *N*-methyltransferase expression was also elevated in the ZDF rat. Homocysteine concentrations were decreased in plasma and kidney, but not in liver, at 12 and 21 weeks. In contrast to hepatic DNA hypomethylation in the type 1 diabetic rat, genomic DNA was hypermethylated at 12 and 21 weeks in the liver of ZDF rats, concomitant with an increase in DNA methyltransferase 1 expression at 21 weeks.

**Conclusions** The pathogenesis of type 2 diabetes in the ZDF rat was associated with tissue and disease stage-specific aberrations of methyl group and homocysteine metabolism, with persistent hepatic global DNA hypermethylation.

## Disciplines

Food Chemistry | Food Science | Human and Clinical Nutrition | Molecular, Genetic, and Biochemical Nutrition | Other Food Science

## Comments

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Diabetes/Metabolism Research and Reviews -

## Tissue-specific alterations of methyl group metabolism and DNA hypermethylation in the Zucker (type 2) diabetic fatty rat

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Keyword primary:	diabetes, ZDF rat, methyl group metabolism, DNA methylation, GNMT
Abstract:	<p><b>Background</b> Altered methyl group and homocysteine metabolism was tissue-specific, persistent, and preceded hepatic DNA hypomethylation in type 1 diabetic rats. Similar metabolic perturbations have been shown in the Zucker (type 2) diabetic fatty (ZDF) rat in the pre- and early diabetic stages, but tissue specificity and potential impact on epigenetic marks are unknown, particularly during pathogenesis.</p> <p><b>Methods</b> ZDF (fa/fa) and lean (+/?) control rats were killed at 12 and 21 wk of age, representing early and advanced diabetic conditions. Blood and tissues were analyzed with respect to methyl group and homocysteine metabolism, including DNA methylation.</p> <p><b>Results</b> At 12 wk, hepatic glycine N-methyltransferase (GNMT), methionine synthase, and cystathionine <math>\beta</math>-synthase (CBS) activity and/or abundance were increased in ZDF rats. At 21 wk, GNMT activity was increased in liver and kidney; however, only hepatic CBS protein abundance (12 wk) and betaine-homocysteine S-methyltransferase mRNA expression (21 wk) were significantly elevated (78 and 100%, respectively). Hepatic phosphatidylethanolamine N-methyltransferase expression was also elevated in the ZDF rat. Homocysteine concentrations were decreased in plasma and kidney, but not in liver, at 12 and 21 wk. In contrast to hepatic DNA hypomethylation in the type 1 diabetic rat, genomic DNA was hypermethylated at 12 and 21 wk in the liver of ZDF rats, concomitant with an increase in DNMT1 expression at 21 wk.</p> <p><b>Conclusions</b> The pathogenesis of type 2 diabetes in the ZDF rat was associated with tissue- and disease stage-specific aberrations of methyl group and homocysteine metabolism, with persistent</p>

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**Tissue-specific alterations of methyl group metabolism and DNA hypermethylation in the  
Zucker (type 2) diabetic fatty rat**

Kelly T. Williams and Kevin L. Schalinske

Running title: Methyl group metabolism in the ZDF rat

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**Abstract**

**Background** Altered methyl group and homocysteine metabolism was tissue-specific, persistent, and preceded hepatic DNA hypomethylation in type 1 diabetic rats. Similar metabolic perturbations have been shown in the Zucker (type 2) diabetic fatty (ZDF) rat in the pre- and early diabetic stages, but tissue specificity and potential impact on epigenetic marks are unknown, particularly during pathogenesis.

**Methods** ZDF (*fa/fa*) and lean (+/?) control rats were killed at 12 and 21 wk of age, representing early and advanced diabetic conditions. Blood and tissues were analyzed with respect to methyl group and homocysteine metabolism, including DNA methylation.

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**Conclusions** The pathogenesis of type 2 diabetes in the ZDF rat was associated with tissue- and disease stage-specific aberrations of methyl group and homocysteine metabolism, with persistent hepatic global DNA hypermethylation.

**Key Words:** diabetes; ZDF rat; methyl group metabolism; DNA methylation; GNMT

## Introduction

It is estimated that over 12% of the U.S. population is afflicted with diabetes, with type 2 diabetes representing 95% of all cases [1]. Diabetes has recently been identified as a condition that can profoundly alter methyl group and homocysteine metabolism [2,3]. There are three primary pathways involved in the metabolism of methyl groups and homocysteine: transmethylation, remethylation via folate/B<sub>12</sub>-dependent or –independent mechanisms, and transsulfuration (Figure 1). For transmethylation reactions, methionine is activated to *S*-adenosylmethionine (SAM), which serves as the universal methyl group donor for numerous methyltransferases, resulting in the methylation of substrates such as nucleic acids, lipids, and proteins. All SAM-dependent methyltransferase reactions result in the generation of homocysteine, which can be remethylated back to methionine using methyl groups donated by either 5-methyltetrahydrofolate (5-CH<sub>3</sub>-THF) or betaine. For folate-dependent remethylation, the B<sub>12</sub>-dependent enzyme methionine synthase (MS) utilizes a methyl group from 5-CH<sub>3</sub>-THF, whereas betaine-homocysteine *S*-methyltransferase (BHMT) catalyzes the folate-independent remethylation of homocysteine using betaine, a methyl group donor derived from the oxidation of choline. Alternatively, homocysteine can be catabolized through the transsulfuration pathway to cysteine, beginning with its irreversible conversion to cystathionine by cystathionine β-synthase (CBS).

We, and others, have previously shown that an acute diabetic condition alters methyl group and homocysteine metabolism [4-9]. Diabetes induced hepatic protein abundance and activity of glycine *N*-methyltransferase (GNMT) and phosphatidylethanolamine *N*-methyltransferase (PEMT), enzymes that have important roles in regulation of methyl groups and homocysteine. GNMT is proposed to be a regulator of the transmethylation potential via

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maintenance of the SAM:S-adenosylhomocysteine (SAH) ratio, whereas PEMT catalyzes the sequential trimethylation of phosphatidylethanolamine to phosphatidylcholine and is purported to be the greatest consumer of SAM-derived methyl groups [10]. Induction of BHMT and CBS are also consistently observed in diabetic or glucocorticoid-treated conditions [4,5,7-9,11,12], whereas the effect of diabetes on MS is not consistent. Taken together, these metabolic alterations result in hypohomocysteinemia in the early diabetic condition. Importantly, similar metabolic aberrations have been observed in both type 1 and type 2 diabetic rat models, as well as diabetic patients [4,5,7-9,13,14]

Progression of diabetes is associated with the development of secondary complications, including cardiovascular disease, nephropathy, neuropathy, and retinopathy. As renal dysfunction in diabetic patients develops, there is a shift from hypo- to hyperhomocysteinemia and suppression of 5,10-methylenetetrahydrofolate reductase (MTHFR) activity that correlates with severity of nephropathy [14,15]. Type 2 diabetics with nephropathy also have decreased flux through the transsulfuration pathway, which likely contributes to a reduction in metabolic homocysteine clearance [16]. Using the streptozotocin (STZ)-treated rat, we have reported tissue-specific and sustained alterations of hepatic methyl group/ homocysteine metabolism appeared to result in a functional methyl deficient condition, as evidenced by hepatic genomic DNA hypomethylation [7]).

The progression of type 2 diabetes from prediabetes to the early diabetic state in the Zucker (type 2) diabetic fatty (ZDF) rat with respect to methyl group and homocysteine metabolism has been reported [9]; however, the more advanced diabetic condition has not been fully characterized. Moreover, the consequences of perturbed methyl group metabolism on DNA methylation has not been examined as a function of type 2 diabetes progression. Similar



alterations in methyl group metabolism have been reported when comparing type 1 and type 2 diabetes [14]. Therefore, we hypothesized that ZDF rats with advanced type 2 diabetes would have perturbed methyl group and homocysteine metabolism, including chronic upregulation of GNMT, and develop a methyl-deficient condition similar to type 1 diabetes [7]. Furthermore, due to the functional methyl deficiency, we hypothesized that the animals would have abnormal DNA methylation and expression of epigenetic regulatory proteins, similar to that observed in the STZ-diabetic rat [7] or rats fed methyl-deficient diets [16]. To date, abnormal DNA methylation as a function of type 2 diabetes has not been examined or reported.

## Materials and Methods

### Chemicals and reagents

Reagents were obtained as follows: 3T3 fully methylated DNA, *BssHII*, *HpaII*, and *MspI* restriction enzymes, New England Biolabs, Inc. (Ipswich, MA); 5- $^3\text{H}$ -dCTP, MP Biomedicals (Solon, OH); enhanced chemiluminescence Western blotting detection reagents and 5- $^{14}\text{C}$ -methyl-THF, Amersham/GE Healthcare (Piscataway, NJ); CBS antibody (H-300, sc-67154), goat anti-rabbit and goat anti-chicken horseradish peroxidase secondary antibodies, Santa Cruz Biotechnology, Inc (Santa Cruz, CA); and *S*-adenosyl-L- $^3\text{H}$ -methionine, Perkin Elmer Life Sciences (Waltham, MA). All other reagents were of analytical grade.

### Animals

All animal protocols were approved by and conducted in accordance with the guidelines set forth by the Iowa State University Institutional Animal Care and Use Committee. For the first study, male rats were obtained at 11 wk of age, allowed to acclimate for 1 wk, and killed at 12 wk of age. For the second study, male rats were obtained at 10 wk of age and killed at 21 wk of age.

Each study utilized six lean (ZDF/Gmi +/?) and six ZDF (ZDF/Gmi *fa/fa*) rats (Charles River Laboratories, Wilmington, MA) kept in individual cages with a 12-h light-dark cycle and given *ad libitum* access to water and Purina 5008 diet. Following a 12-h fast, rats were anesthetized by intraperitoneal injection with ketamine: xylazine (90: 10 mg/kg body weight, respectively), whole blood samples were collected by cardiac puncture, and blood glucose levels were immediately assessed using a glucometer. Plasma and serum samples were obtained by centrifugation and stored at -20°C. Serum insulin levels were analyzed by Rat/Mouse Insulin ELISA Kit (Linco, Inc., St Charles, MO). Tissues were rapidly removed and a portion of the liver was immediately homogenized for preparation of cytosolic extracts as previously described [7]. The remainder of the liver and all other tissues were frozen in liquid nitrogen and stored at -70°C. The total soluble protein concentration of extracts was determined using the Bradford assay with Coomassie Protein Plus Reagent (Thermo Scientific).

**Enzyme assays**

The enzymatic activity of GNMT, PEMT, and MS were determined using radioisotopic assays as previously described [5-7,17,18]. Briefly, GNMT and MS activity in the liver, kidney, and heart were determined by incubation of cytosolic extracts with reaction mixes containing *S*-adenosyl-L-[<sup>3</sup>H-*methyl*]-methionine or 5-[<sup>14</sup>C]-methyl-THF, respectively. After terminating the reaction, the unreacted radiolabeled substrate was removed using activated charcoal or anion exchange resin, respectively. Using microsomal extracts, hepatic PEMT activity was assessed by determining the incorporation of *S*-adenosyl-L-[<sup>3</sup>H-*methyl*]-methionine into the lipid fractions. Liquid scintillation counting was used to determine radiolabel incorporation for all assays.

**Immunoblotting**

Relative protein abundance was determined using methods previously described [7] with minor modifications. Proteins were separated using SDS-PAGE and subsequently transferred to nitrocellulose. For assessment of GNMT protein abundance, blots were incubated with an affinity-purified chicken antibody (Aves Labs, Inc., Tigard, OR) directed against the peptide sequence KER WNR RKE PAF DK (GNMT residues #97-110) diluted 1:40,000 in TTBS/ 10% BSA. CBS primary antibody was prepared 1:400 in TTBS/ 10% BSA, and goat anti-chicken or anti-rabbit horseradish peroxidase secondary antibodies were diluted 1:5,000 in TTBS. Following incubation with chemiluminescence reagents and subsequent exposure to film, relative protein abundance was quantified by densitometric analysis with QuantityOne software (Bio-Rad Laboratories, Hercules, CA).

### DNA methylation

Using the method of Pogribny et al [19] and as described previously [7], digestion of DNA followed by cytosine extension was used for assessment of DNA methylation status in liver, kidney, and heart tissues. Global and CpG island DNA methylation were determined by digesting 1.0 µg DNA using the methylation-sensitive restriction enzymes *HpaII* and *BssHII*, respectively. For the cytosine extension assay, a reaction mixture of the DNA digest, 10X PCR Buffer II (without MgCl<sub>2</sub>), 25 mM MgCl<sub>2</sub>, 0.5 U Amplitaq DNA Polymerase (Applied Biosystems), and [<sup>3</sup>H]-dCTP was incubated at 55°C for 1 h. Following incubation, samples were applied to Whatman DE-81 ion exchange filter paper and washed in 0.5 mM sodium phosphate buffer (pH 7.0) three times, dried, and <sup>3</sup>H incorporation was assessed using liquid scintillation counting. Samples were run in duplicate and reactions using either *MspI*-digested DNA or mouse 3T3 fully-methylated DNA with the appropriate restriction enzyme were used as controls.

The degree of genomic methylation fell within the expected range of 70-90% [20] compared to the *MspI* control.

**Real-time RT-PCR**

A 0.1 g sample of frozen liver was preserved in 1.0 mL RNALater-ICE (Ambion, Inc, Austin, TX) and subsequently used for RNA isolation using Trizol Reagent (Invitrogen, Carlsbad, CA) with TURBO DNA-free (Ambion, Inc.) DNase treatment. The reverse-transcription assay was performed using iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) and the resulting cDNA was diluted 50-fold for use in the PCR reaction with iQ SYBR Green Supermix (Bio-Rad Laboratories). The specific primers used for RT-PCR (Table 1) were designed using PrimerQuest (Integrated DNA Technologies, Inc., Coralville, IA). Samples were run in duplicate, data were normalized to 18S control, and results were analyzed using the comparative  $C_t$  method.

**Statistical analysis**

For each time point, the mean values of each treatment group (lean vs. ZDF) were subjected to a Student's *t* test (SigmaStat, SPSS, Chicago, IL). A Mann-Whitney rank sum test was used when normality or equal variance test failed. Differences were considered significant when  $P \leq 0.05$  using a one-tailed test.

**Results**

**Confirmation of a diabetic condition in ZDF rats**

All ZDF rats were hyperglycemic (blood glucose:  $26.2 \pm 1.9$  and  $30.2 \pm 0.8$  mM) compared to control values (blood glucose:  $12.1 \pm 0.9$  and  $17.8 \pm 1.1$  mM) at 12 and 21 wk, respectively. Likewise, serum insulin levels were elevated at 12 and 21 wk ( $0.63 \pm 0.10$  and  $0.81 \pm 0.13$

ng/mL) compared to values ( $0.32 \pm 0.03$  and  $0.44 \pm 0.02$  ng/mL), indicating a hyperinsulinemic state.

### **Perturbation of methyl group metabolism regulatory proteins in the ZDF rat**

Diabetes produced tissue- and time-specific changes in the activity (Table 2) and expression (Table 3) of a number of key enzymes involved in methyl group metabolism. GNMT and PEMT are key proteins in the regulation of methyl group and homocysteine balance. The activity and protein abundance of GNMT were elevated in the liver of ZDF rats compared to lean controls at both 12 and 21 wk of age (Table 2). In support of this finding, GNMT mRNA abundance was also increased in the liver at 21 wk (Table 3). Renal GNMT activity was increased at 21 wk (Table 2); however, a **corresponding** increase in renal GNMT protein abundance was not observed, suggesting possible post-translational regulation of GNMT activity in the diabetic rat kidney. Contrary to our previous results using the type 1 diabetic rat, hepatic PEMT activity was unchanged in the ZDF rat at 12 wk and enzyme activity was decreased at 21 wk (lean vs. ZDF:  $88 \pm 10$  vs.  $62 \pm 8$  pmol/min·mg protein,  $P = 0.035$ ). However, similar to the increase in PEMT activity noted in the type 1 diabetic rat (6), there was a trend ( $P < 0.10$ ) towards increased hepatic PEMT mRNA abundance at 12 wk and a statistically significant 73% increase in PEMT mRNA abundance in ZDF rat liver at 21 wk (Table 3).

Removal of homocysteine via folate/B<sub>12</sub>-dependent and -independent remethylation (MS and BHMT, respectively), or catabolism by the transsulfuration pathway (CBS), was significantly altered in the ZDF rat **that was highly dependent on age and was tissue-specific**. In agreement with the type 1 diabetic rat (4), BHMT mRNA abundance was increased in the ZDF rat liver at 21 wk (Table 3). In the early diabetic condition (12 wk), hepatic MS activity was significantly increased (Table 2), supported by a similar trend for MS mRNA abundance (Table

3); however, no significant differences were observed in the liver at 21 wk, or in renal MS activity. CBS did not show any changes in mRNA abundance (Table 3), but there was a tissue-specific effect on protein abundance (Table 2). Similar to observations from the STZ-diabetic rat (7), CBS protein abundance was greater in the liver, whereas it was decreased in the kidney when ZDF rats were compared to lean controls at 12 wk. At 21 wk, hepatic CBS abundance was reduced in the ZDF rat, and no significant difference was observed in renal CBS protein abundance between ZDF and lean rats. For heart tissue, MS activity was increased and CBS abundance was decreased 12% in ZDF rats relative to controls (Table 2) at 21 wk. In both the early and advanced diabetic conditions, cardiac GNMT protein abundance was similar between diabetic rats and controls; however, no appreciable cardiac GNMT activity was detected.

**Perturbation of homocysteine balance in the ZDF rat**

Plasma total homocysteine concentrations were decreased 67 and 54% in ZDF rats at 12 and 21 wk, respectively, compared to the lean controls (Table 4). There were no changes in the hepatic concentration of homocysteine at either time point. However, homocysteine concentrations in the kidney reflected the changes in plasma homocysteine, exhibiting a significant decrease of 63 and 49% in the ZDF kidney at 12 and 21 wk, respectively.

**DNA hypermethylation and upregulation of DNMT1 in the type 2 diabetic rat liver**

In contrast to the development of hepatic global DNA hypomethylation in the advanced STZ-diabetic rat (7), global DNA hypermethylation was characteristic of ZDF rat liver at both 12 and 21 wk (Figure 2). This effect was specific to genomic DNA in the liver; global DNA changes were not observed in the kidney (Figure 2) and heart, nor in CpG island methylation status as measured by the overall incorporation of [<sup>3</sup>H]-dCTP. DNMT1 mRNA abundance was increased 95% in the ZDF rat liver at 21 wk (Table 3). No significant changes were observed in the

mRNA abundance of other key nuclear proteins involved in DNA methylation, including DNMT 3a and 3b, as well as MBD 1,2,3,4 and MeCP2 (data not shown).

## Discussion

Here, we demonstrate that during the progression of type 2 diabetes in the ZDF rat there are tissue-specific alterations of methyl group and homocysteine metabolism consistent with type 1 diabetes [4-7], accompanied by concomitant changes in epigenetic marks, namely the methylation of DNA. However, unlike the effects of the chronic type 1 diabetic condition in which we observed hepatic genomic DNA hypomethylation [7], genomic DNA was hypermethylated in the ZDF rat liver, even in the early (12 wk) stages of the disease. In early diabetes, both ZDF and type 1-diabetic rat models have been shown to exhibit similar perturbations in hepatic methyl group and homocysteine metabolism [4-9], consistent with our observations with ZDF rats at 12 wk of age. The net effect of these metabolic perturbations may result in the loss of methyl groups via induction of GNMT and an increase in the flux through the transsulfuration pathway, although this was not directly measured. This might be anticipated based on the abundance of evidence supporting GNMT as a regulator of the SAM:SAH ratio [22], and the known induction of CBS expression and activity by glucocorticoids and SAM, respectively [23,24]. The upregulation of CBS may be a compensatory mechanism for self-protection of the liver and kidney against the oxidative stress associated with a diabetic condition. CBS activity has been shown to be enhanced under conditions of oxidative stress [25] and the antioxidant glutathione, a by-product of the transsulfuration pathway, has been reported to be depleted in diabetic tissues [26].



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It appears that the pathogenesis of type 2 diabetes from 12 to 21 wk results in further alterations in methyl group metabolism. The induction of GNMT at 21 wk was not as robust as that observed for 12 wk with only a 19 and 8% increase in activity and protein abundance, respectively. However, there was a significant increase in BHMT mRNA abundance at 21 wk that was not observed in the early (12 wk) diabetic condition. For diabetes, increases in BHMT mRNA abundance have previously been associated with increased BHMT activity [12], suggesting enhanced homocysteine remethylation. Interestingly, PEMT was upregulated at the mRNA level, whereas PEMT activity was decreased at 21 wk. Taken together, a decrease in production of homocysteine by PEMT and GNMT, combined with an increase in BHMT activity, might be expected to contribute to the hypohomocysteinemia that persisted in the advanced diabetic state despite no change in CBS protein abundance. Others have shown that diabetes also increases homocysteine catabolism via transsulfuration [9,12].

The response of the kidney to a diabetic condition appears to be less stringently regulated compared to the liver. Whereas hepatic enzymes relevant to methyl group and homocysteine metabolism appear to be regulated at a transcriptional and/or translational level during diabetes progression, there was a lesser impact on these enzymes in the kidney. Thus, a diabetic condition appears to have a direct impact on hepatic methyl group metabolism via alterations in gene expression, whereas renal methyl group metabolism may reflect the diminished supply of methyl groups from the liver. Renal GNMT activity was only increased in the more advanced diabetic state (21 wk) and there were not concomitant increases in protein abundance. We have previously established that GNMT regulation is tissue-specific in response to retinoids [26], as well as diabetes [7]. Aside from the increase in GNMT activity, there were no observed alternations of other methyl group-related enzymes, nor was DNA methylation status affected in



the kidney. House et al [27] also found a lack of effect of either glucagon treatment or STZ-diabetes on renal methyl group metabolism, whereas Jacobs et al [8] reported that renal MS and MTHFR activities were suppressed by a type 1 diabetic condition.

Homocysteine balance within a tissue, and subsequently the circulation, reflects the collective metabolic routes linked to its production, remethylation, and catabolism. The liver is unique in that it contains the full complement of pathways involved in methyl group metabolism. The kidney also exhibits all of the pathways [28]; however, BHMT is expressed at low levels in the rat kidney compared to human tissue [29] and MS activity was found not to be quantitatively significant [28,30], resulting in transsulfuration being the primary route for removal of homocysteine in the kidney. Homocysteine levels in the ZDF liver were not different from controls at 12 or 21 wk, despite decreased homocysteine concentrations in both the plasma and kidney. It is unlikely that the low levels of homocysteine in the kidney are due to increased urinary excretion, because this was shown not to be a significant route for homocysteine removal in healthy or STZ-diabetic kidneys [8,30]. However, this may be different for the ZDF rat, where glomerular filtration rate was elevated at 3 months of age [31]. Thus, the reduction in renal and plasma homocysteine concentrations may be the result of increased excretion in the ZDF rat model. The kidney has been shown to contribute significantly to the removal of homocysteine from the circulation [32] and data suggests it contains adequate CBS to compensate for acute or chronic increases in plasma homocysteine [28]. Taken together, it appears that the persistent hypohomocysteinemia observed in type 2 diabetes rat models might be due to an irreversible loss of homocysteine via the renal transsulfuration pathway and/ or excretion. In contrast, homocysteine may be remethylated to methionine in the kidney owing to

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a markedly elevated expression of BHMT in humans compared to the rat, thereby conserving the homocysteine moiety and contributing to the eventual development of hyperhomocysteinemia.

The observation of global DNA hypermethylation in the livers of ZDF rats was particularly intriguing and completely divergent from our previous observations in the type 1 diabetes [7]. Although DNA hypomethylation has been shown to be associated with a number of pathological conditions, hypermethylation of DNA can also have deleterious effects. DNA methylation at cytosine residues within CpG dinucleotides is an important modulator of chromatin structure, repressor of transposable elements, and regulator of gene expression [33,34]. The CpG sequence represents only 1-4% of the genome and this is proposed to be due to selection against the sequence due to its high potential for deleterious effects [35]. An increase in the levels of 5-methylcytosine elevates the chance of point mutations throughout the genome by spontaneous deamination [36], whereby an unmethylated cytosine base is deaminated to uracil and deamination of methylated cytosine produces thymine. If undetected by DNA repair enzymes prior to replication, the transition mutation will be maintained and carried on to daughter cells, thereby contributing to genomic instability. Moreover, induction of genomic DNA hypermethylation owing to overexpression of DNMT1 was associated with chromosomal overcondensation, as well as chromosomal rearrangement and misalignment of sister chromatids [37], which could be expected to alter chromosomal segregation during cell division. Genomic hypermethylation has been associated with increased methylation at histone 3 lysine 9 [37], a finding that was also observed in lymphocytes from type 1 diabetic patients and associated with the promoter regions of a number of genes involved in inflammation, which may contribute to the development of secondary complications [38]. Likewise, one of the few reports of genomic DNA hypermethylation in clinical studies found that leukocytic DNA hypermethylation was

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3 associated with increased inflammation and increased mortality in patients with chronic kidney  
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5 disease [39].  
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8 The pathophysiology of ZDF rats is characterized by obesity, hyperglycemia,  
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10 hyperinsulinemia, hyperlipidemia, and hypertension [40]). ZDF rats develop hyperinsulinemia  
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12 and insulin resistance before 7 wk of age, with an incremental drop in elevated insulin levels  
13  
14 around 8 wk of age, and at 12 wk of age blood glucose levels rise, resulting in animals becoming  
15  
16 overtly diabetic [41]. Although insulin levels continue to decrease due to pancreatic  $\beta$ -cell  
17  
18 exhaustion, ZDF rats may remain hyperinsulinemic relative to lean controls well into the  
19  
20 advanced diabetic stage [21], as was observed in this study. In contrast, chemically-induced type  
21  
22 1 diabetes also results in hyperglycemia, but is characterized by a lack of insulin production.  
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24 Treatment of rats or hepatic cell lines with glucocorticoids, such as dexamethasone or  
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26 triamcinolone, has also been shown to induce expression of methionine adenosyltransferase  
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28 (MAT), GNMT, BHMT, and CBS at the transcriptional level and insulin administration has been  
29  
30 shown to prevent these alterations in both rats and/or cell lines [4,11,12,42,43]. Interestingly,  
31  
32 insulin stimulates transmethylation, and transsulfuration flux in healthy individuals [44]. Insulin  
33  
34 treatment of untreated HepG2 hepatocarcinoma cells was also capable of inducing MAT activity  
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36 [45]; thus, insulin likely plays a role in the metabolic perturbations observed in the early type 2  
37  
38 diabetic state. Treatment of HepG2 cells with glucose also resulted in stimulation of MAT  
39  
40 activity and induced genomic hypermethylation [45]. Several recent cell culture studies that  
41  
42 were designed to mimic hyperglycemia have reported histone modifications which persist even  
43  
44 after restoration of normal glucose concentrations [38,46-48]. Based on genome-wide profiling  
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46 of specific histone modifications, these studies implicate hyperglycemia in the epigenetic  
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48 regulation of pathways involved in signal transduction, oxidative stress, immune function, and  
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inflammation. The bulk of evidence indicates that aberrations of methyl group metabolism and epigenetic regulation are likely due to the combination of hyperglycemia, a lack of insulin or insulin resistance, and elevated counter-regulatory hormones. In support of this, we have recently demonstrated that insulin administration prevents many of the alterations in methyl group and homocysteine metabolism, including the hypomethylation of DNA, in a type 1 diabetes rat model [49]. The differential response in our observed changes in methyl group metabolism as a function of type 1 vs. type 2 diabetes may reside in differences in metabolic signals that are characteristic of each diabetic state. In both human patients and animal models, C peptide is increased in type 2 diabetes, but markedly decreased in type 1 diabetes [49]. C peptide has been shown to exhibit a number of insulinomimetic properties, be involved in cell signaling, and has been implicated in the development of vascular inflammation and atherosclerosis in type 2 diabetes [49]. However, the actions of C peptide in type 2 diabetics are largely uncharacterized and may provide an opportunity for investigation into the pathological differences between type 1 and type 2 diabetes, particularly with respect to methyl group metabolism.

In summary, we have shown that the metabolism of methyl groups and homocysteine were altered in a tissue-specific manner during the pathogenesis of type 2 diabetes in the ZDF rat. Although there are many commonalities in the regulation of methyl group and homocysteine metabolism in type 1 and type 2 diabetes, the impact on epigenetic regulation (i.e., DNA methylation) was divergent between the two diabetic conditions. Abnormalities of enzymatic regulation and key metabolite concentrations have been observed as early as 5 wk of age [9] and we have shown hepatic DNA hypermethylation was evident at 12 wk of age in ZDF rats. Data are lacking prior to these time points; thus, it is unclear when abnormalities in methyl group metabolism and epigenetic regulation are initiated in the ZDF rat. However, it is evident that

1  
2  
3 perturbations of methyl group metabolism and aberrant DNA methylation represent early events  
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5 in the pathogenesis of type 2 diabetes with potentially persistent effects owing to the stable  
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7 nature of epigenetic marks. The role of DNA methylation in diabetes and its potential relation to  
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9 diabetic complications, such as nephropathy [50], is clearly an important and emerging field.  
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18  
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21 Huntsville, AL.  
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### 29 **Conflict of Interest**

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31 None.  
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**Figure Legends**

**Figure 1.** Methyl group and homocysteine metabolism. Enzymes are shown in black boxes, whereas vitamin substrates and/or cofactors are shown in gray boxes. Abbreviations are: betaine-homocysteine *S*-methyltransferase (BHMT); cystathionine  $\beta$ -synthase (CBS); dimethylglycine (DMG); methionine synthase (MS); methyltransferases (MTs); 5,10-methylene-THF reductase (MTHFR); *S*-adenosylhomocysteine (SAH); *S*-adenosylmethionine (SAM); tetrahydrofolate (THF); and methyl acceptor (X). Key SAM-dependent methyltransferases include: glycine *N*-methyltransferase (GNMT); phosphatidylethanolamine *N*-methyltransferase (PEMT); and DNA methyltransferase 1 (DNMT1). These three methyltransferases respectively catalyze the conversion of glycine to sarcosine, phosphatidylethanolamine to phosphatidylcholine, and cytosine-DNA to 5-methyl-cytosine-DNA . In addition to THF, this series of interrelated pathways are dependent on a number of other B-vitamins, including riboflavin (B<sub>2</sub>), vitamin B<sub>6</sub>, and vitamin B<sub>12</sub>.

**Figure 2.** Hepatic and renal methylation status of genomic DNA in lean (open bars) and ZDF (black bars) rats at (A) 12 and (B) 21 wk of age. Using the method of Pogribny et al [19], incorporation of [<sup>3</sup>H]-dCTP is inversely related to the degree of endogenous methylation. Values are mean  $\pm$  SEM (n = 6). \*Different from control, *P*  $\leq$  0.05.

**Table 1. Real-time RT-PCR primers**

Target	Primers (5' → 3')
GNMT	F*: ACA ACA AAG CCC ACA TGG TAA CCC R: AGC CGA AAC TTA CTG AAG CCA GGA
PEMT	F: TGT GCT CTC CAG CTT CTA TGC ACT R: AGG GAA ATG TGG TCA CTC TGG ACT
MS	F: TTG GCC TAC CGG ATG AAC AAA TGC R: AGC CAC AAA CCT CTT GAC TCC TGT
BHMT	F: ATC TGG GCA GAA GGT CAA TGA AGC R: TGA CTC ACA CCT CCT GCA ACC AAT
CBS	F: AAC ATG TTG TCC TCC CTG CTT GCT R: TCG GCT TGA ACT GCT TGT AGA GGA
DNMT1	F: TGT GGC AAG AAG AAA GGT GGC AAG R: TGG ATG GAC TTG TGG GTG TTC TCA
DNMT3a	F: AGA GTG TCT GGA ACA CGG CAG AAT R: TGC TGG TCT TTG CCC TGC TTT ATG
DNMT3b	F: TGC GCC TGC AAG ACT TCT TCA CTA R: TGC AGG AAT CGC TGG GTA CAA CTT
MBD1	F: CCT GCA CCT TTG TGC TGT GAG AAT R: CAG TCT TTG CAC AAT GTC CTG CGT
MBD2	F: TCA GAA GTA AAC CAC AGC TGG CGA R: ACT AGG CAT CAT CTT GCC GCT TCT

MBD3	F:	GAA GAA GTT TCG CAG CAA GCC ACA
	R:	CAT CTT TCC CGT GCG GAA ATC GAA
MBD4	F:	AGC TAA ACC TCA GGA CAC GAA GCA
	R:	TTG GAC AGGCTG TTG CTA TCT GGA
MeCP2	F:	GCA GCA GCA TCA GAA GGT GTT CAA
	R:	TGC TTG GAA AGG CAT CTT GAC GAG
18S	F:	GAA CCA GAG CGA AAG CAT TTG CCA
	R:	ATG GTC GGA ACT ACG ACG GTA TCT

\*F denotes forward primer, R denotes reverse primer.

**Table 2. Tissue-specific activity and abundance of enzymes regulating methyl group and homocysteine metabolism in lean (+/?) and ZDF (*fa/fa*) rats at 12 and 21 wk of age**

	Tissue	12 wk		21 wk	
		Lean	ZDF	Lean	ZDF
GNMT Activity (pmol/(min • mg protein))	Liver	305 ± 17	381 ± 7*	273 ± 8	324 ± 13*
	Kidney	45 ± 1	43 ± 3	54 ± 5	73 ± 3*
	Heart	n.d.	n.d.	n.d.	n.d.
GNMT Abundance Relative to control	Liver	1.00 ± 0.14	1.65 ± 0.10*	1.00 ± 0.03	1.08 ± 0.02*
	Kidney	1.00 ± 0.10	0.80 ± 0.11	1.00 ± 0.07	0.98 ± 0.07
	Heart	1.00 ± 0.01	0.99 ± 0.01	1.00 ± 0.04	0.99 ± 0.05
MS activity (pmol/(min • mg protein))	Liver	416 ± 40	498 ± 21*	541 ± 29	562 ± 40
	Kidney	886 ± 47	976 ± 15	809 ± 34	746 ± 22
	Heart	375 ± 13	359 ± 7	286 ± 20	343 ± 22*
CBS Abundance Relative to control	Liver	1.00 ± 0.09	1.27 ± 0.05*	1.00 ± 0.09	0.78 ± 0.06*
	Kidney	1.00 ± 0.11	0.67 ± 0.11*	1.00 ± 0.08	1.02 ± 0.07
	Heart	1.00 ± 0.14	0.81 ± 0.16	1.00 ± 0.03	0.88 ± 0.05*

Data are means ± SEM (n = 4-6). The abbreviation n.d. indicates activity was not detected.

Asterisks (\*) indicate a significant difference from control,  $P \leq 0.05$ .

**Table 3. Hepatic mRNA abundance of enzymes regulating methyl group supply and utilization in lean (+/?) and ZDF (*fa/fa*) rats at 12 and 21 wk of age.**

Target	12 weeks, mean fold induction		21 weeks, mean fold induction	
	Lean	ZDF	Lean	ZDF
GNMT	1.00 ± 0.55	1.03 ± 0.23	1.00 ± 0.13	1.36 ± 0.16*
PEMT	1.00 ± 0.40	3.31 ± 1.20	1.00 ± 0.18	1.73 ± 0.21*
MS	1.00 ± 0.15	1.91 ± 0.58	1.00 ± 0.27	1.30 ± 0.24
BHMT	1.00 ± 0.49	1.64 ± 0.47	1.00 ± 0.22	2.06 ± 0.54*
CBS	1.00 ± 0.54	0.77 ± 0.29	1.00 ± 0.28	1.10 ± 0.20
DNMT1	1.00 ± 0.23	0.97 ± 0.33	1.00 ± 0.29	1.95 ± 0.37*

Data are means ± SEM (n = 4-6). Asterisks (\*) indicate a significant difference from control, *P* ≤ 0.05.

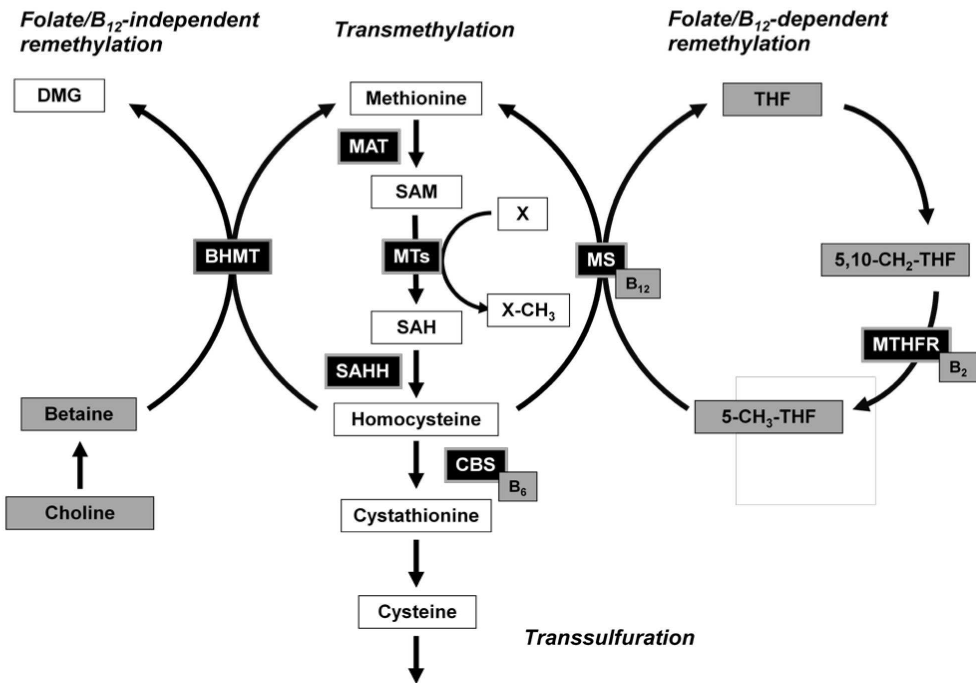


**Table 4. Fasted plasma and tissue concentrations of total homocysteine in lean (+/?) and ZDF (*fa/fa*) rats.**

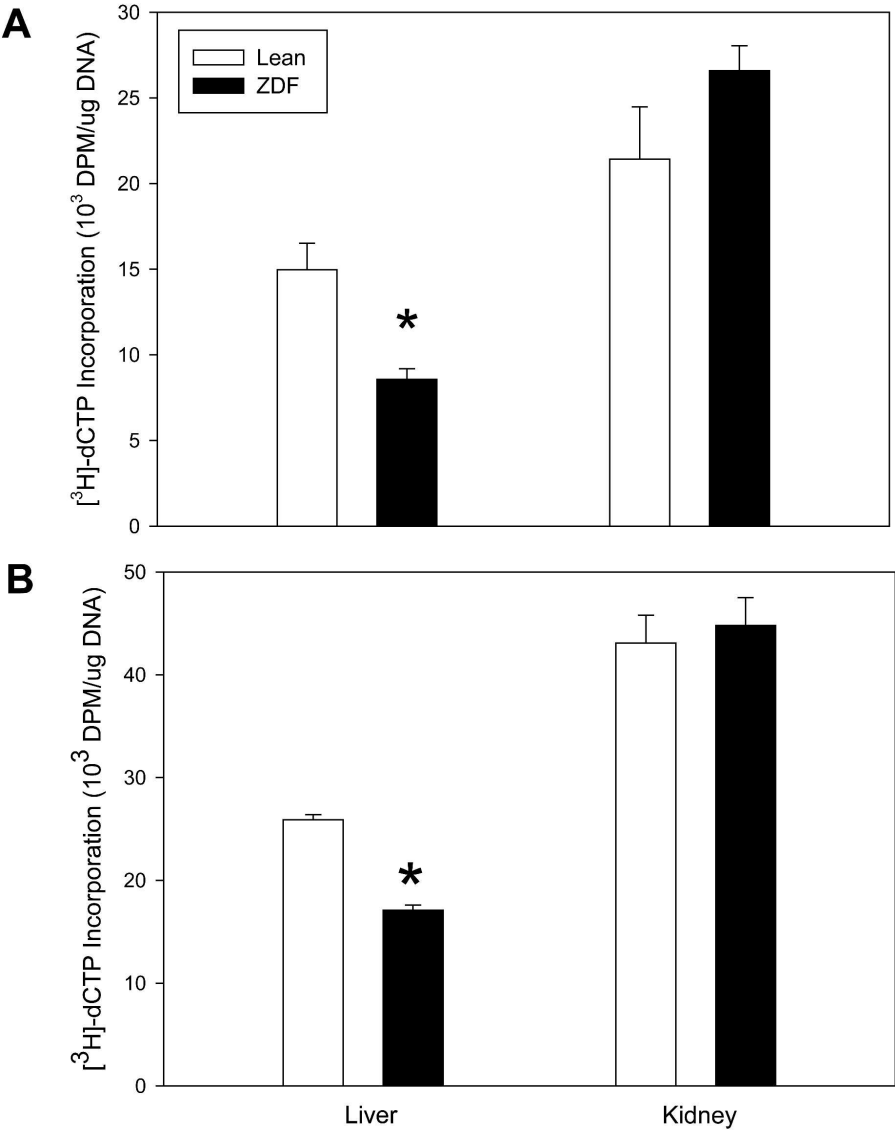
Sample source	Age (wk)	Total Homocysteine ( $\mu$ M plasma or $\mu$ mol/g tissue)	
		Lean	ZDF
Plasma	12	$6.8 \pm 0.3$	$2.2 \pm 0.1^*$
	21	$3.6 \pm 0.3$	$1.6 \pm 0.1^*$
Liver	12	$155 \pm 22$	$166 \pm 10$
	21	$168 \pm 7$	$203 \pm 20$
Kidney	12	$11.0 \pm 4.1$	$4.1 \pm 0.7^*$
	21	$6.3 \pm 0.9$	$3.2 \pm 1.1^*$

Data are means  $\pm$  SEM (n = 4-6). Asterisks (\*) indicate a significant difference from control,  $P \leq 0.05$ .

Figure 1



169x155mm (600 x 600 DPI)



169x208mm (600 x 600 DPI)